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The interaction of the estrogen receptor with mononucleosomes

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Abstract

To directly activate specific gene expression, the estrogen receptor (ER) must bind to estrogen receptor response elements (EREs) in the context of nucleosomes. In order to investigate the interaction of the ER with mononucleosomes, we developed a mononucleosome gel shift assay. A 164 bp high specific activity [^{32}P]probe DNA (32 bp consensus ERE with flanking regions separated by 23 nucleotides from an artificial nucleosome positioning sequence) was prepared. Nuclear extracts from MCF-7 cells or recombinant human ERα were incubated with the labeled ERE ± excess ERE. A retarded band was seen which was completely obliterated with excess ERE, confirming the specificity of binding. This probe was then used to make reconstituted mononucleosomes by sequential dilution of a high salt histone preparation. The nucleosomes were purified by sucrose density gradients and footprinting analysis was performed to demonstrate that the mononucleosomes were rotationally phased as seen by a periodic digestion pattern (10 bp) of the nucleosomes versus ERE. Nucleosomes were incubated with nuclear extracts containing ER or recombinant ERα. Dose dependence in the shift of the mononucleosomes with increasing concentrations of ER was observed. Specificity was demonstrated in experiments with excess ERE and anti-ER antibody. Footprinting analysis was also performed. We also determined that addition of high mobility group protein-2 (HMGB-2, a protein closely related to HMGB-1) with the ER increased the interaction of ER with mononucleosomes. These studies will allow us to address the interactions of ER with core histones containing a multiplicity of variants and modifications in nucleosomal structure.
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1. Introduction

Although binding of steroid receptors to DNA is recognized as necessary for induction of many biological responses to steroid hormones, it is now generally accepted that chromosomal protein interaction is also necessary. Steroid hormone receptors have been reported to bind various histones with high affinity (Kallos et al., 1981; Kiyotaka et al., 1989) and nucleosomes can regulate the interaction of steroid receptors with steroid response elements (Pham et al., 1992; Pina et al., 1990). Thus, the initial stage of gene activation is linked with the interaction of trans-acting factors with nucleosomes. The structure and function of the nucleosome itself and its component parts are much more important than merely functioning as DNA packaging proteins. The nucleosome core particle consists of a histone octamer (two H2A, H2B dimers and one H3–H4 tetramer) and 146–160 bp DNA wrapped approximately two turns around the octamer. The core histones themselves are believed to play a role as signaling molecules in modulation of gene activity by repressing or activating gene transcription (Pazin and Kodonaga, 1997; Turner, 1998).

Our laboratory has been particularly interested in the role of modified histones in the binding of the estrogen receptor to DNA (Ruh et al., 1996; Ruh et al., 1999). The present study was initiated to determine the interaction of the estrogen receptor (ER) when the estrogen response element (ERE) is present in mononucleosomal structure. Transcriptional activators, like the estrogen receptor, must contend with the constrained structure of the DNA in a nucleosomal structure. Contacts between the DNA and the histones probably compete for interactions necessary for the binding of the ER. In addition, recent studies have suggested that the high mobility group proteins (HMGB-1 and -2), stabilize the interaction of specific steroid receptors to their respective hormone response elements (Romine et al., 1998; Zang

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et al., 1999; Boonyaratanakornkit et al., 1998; Melvin and Edwards, 1999). However, no studies have been reported on the effect of the HMGB proteins on steroid receptor interaction with hormone response elements in nucleosomal structure. Thus, the study of ER interaction with the ERE in nucleosomal configuration in the presence of HMGB proteins, rather than linearized ERE, should allow us in the future to determine the histone modifications that facilitate ER interaction with the ERE in the context of chromatin.

2. Materials and methods

2.1. Materials

17β-Estradiol was obtained from Sigma (St. Louis, MO). The radionucleotide [α-^{32}P]dATP (3,000 Ci/mmol) and the [^{33}P] to label the ddNTPs in the template DNA were purchased from Perkin–Elmer (Boston, MA). Recombinant ERα and the polyclonal anti-ERα antibody were purchased from PanVera (Madison, WI) and Affinity BioReagents (Golden, CO), respectively. The anti-HMGB-2 polyclonal antibody was purchased from Active Motif (Carlsbad, CA). HMGB-2 was generously provided by D. Edwards, University of Colorado.

2.2. Plasmids

The phBlue plasmid containing a c-Myc/Max binding site separated by 23 nucleotides from an artificial nucleosome positioning sequence was obtained from R.E. Kingston (Harvard). The c-Myc/Max sequence was removed by digestion with the restriction enzymes Nco I and Bgl II and the 32 mer ERE from the vitellogenin promoter (Kumar and Chambon, 1988) containing a 5′ Bgl II site and a 3″ Nco I site was inserted to generate the pERE-Blue plasmid. For EMSAs, the 164 bp *Eco R1-BamH1* fragment of phBlue was gel purified and end-labeled using [α-^{32}P]dATP and Klenow. Labeled probe was separated from unincorporated nucleotides by G-50 Quick Spin columns (Roche, Indianapolis, IN).

2.3. Preparation of nuclear extracts

MCF-7 cells were grown as previously described (Ruh et al., 1999). To prepare MCF-7 cell nuclear extracts, cells were treated with 20 nM estradiol-17β for 2 h at 37 °C and the cells were harvested as previously described (Ruh et al., 1999). Cells were pelleted and resuspended in TEGD buffer (25 mM Tris, 1.5 mM EDTA, 20% glycerol, 1 mM dithiothreitol, pH 7.6), followed by centrifugation at 1000 × *g* for 5 min at 4 °C. The cells were washed twice in the TEGD buffer and then suspended in TED buffer (TEGD buffer minus the glycerol) for 10 min at 4 °C. The cells were homogenized in glass-Teflon homogenizers and the homogenate was centrifuged at 40,000 × *g* for 10 min. The nuclear pellet was incubated with 0.5 M KCl in TEGD for 1 h at 4 °C and centrifuged at 110,000 × *g* for 30 min to obtain the nuclear extracts containing ER. Protein concentration of the MCF-7 nuclear extracts was determined using the Pierce BCA protein assay (Rockford, IL). Nuclear extracts were aliquoted and stored at −80 °C.

2.4. Preparation of core histones

MCF-7 cells (2 × 10^{8} cells) were harvested from plates by scraping into phosphate-buffered saline (PBS) and centrifuged at 1000 × *g* for 5 min at 4 °C. The cell pellets were suspended in 20 ml TCS buffer (10 mM Tris, pH 8.0, 3 mM $CaCl_2$, and 0.25 M sucrose), allowed to sit on ice for 10 min and centrifuged for 5 min at 1000 × *g*. The cell pellets were suspended in 30 ml TCS containing 1% Triton X-100 and centrifuged at 8000 × *g* for 15 min. The combined cell pellet was suspended in 10 ml of TCS plus Triton-X 100, homogenized in a Telflon-glass homogenizer (6 strokes), the volume adjusted to 60 ml with TCS-Triton-X 100 buffer, and centrifuged at 8000 × *g* for 20 min. This step was repeated two times. Nuclei obtained from the above procedure were suspended in 10 ml TCS buffer, homogenized in a Teflon-glass homogenizer (6 strokes), and the amount of DNA quantified (Burton, 1956). To obtain the core histones a modification of the procedure of Stein and Mitchell (1988) was used. Nuclei (1 mg DNA) were suspended in 40 ml 0.35 M NaCl, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, stirred gently for 15 min at 4 °C and pelleted by low speed centrifugation (300 × *g* for 5 min). The nuclear pellet was resuspended in 10 ml 0.6 M NaCl, 50 mM $NaPO_4$ (pH 6.8) to lyse the nuclei and to remove H1. After adding 0.4 g hydroxylapatite to the lyzed nuclei, the volume was adjusted to 40 ml with 0.6 M NaCl, 50 mM $NaPO_4$, 0.2 mM PMSF. The slurry was mixed well and incubated for 10 min on ice. After centrifugation the supernatant containing H1 was discarded. The pellet was washed 5 times in 40 ml of the previous buffer. The pellet was extracted with 2.5 M NaCl, 50 mM $NaPO_4$, pH 6.8 at 4 °C for 15 min to obtain the core histones. The core histones were concentrated to 1 ml using a Millipore Centricon Plus 80 (Ultracel PL10) at 3500 × *g* for 20 min at 4 °C. To check the purity of the core histones, samples (30 ug) were resolved by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R-250.

2.5. Reconstitution of mononucleosomes

The radiolabeled ERE containing 164 bp probe DNA (30 ng) was added to 2.5 μg nonspecific DNA (the DNA probe in phBlue containing c-Myc/Max) and 4 μg core histones in a final concentration of 1.0 M NaCl, 50 mM HEPES, 1 mM EDTA in 10 μl for 20 min at 30 °C. This preparation was sequentially diluted four times to 0.2 M NaCl, using 50 mM HEPES, 1 mM EDTA, 5 mM DTT, 0.5 mM PMSF, final volume 50 μl. Each dilution was incubated for 30 min at 30 °C. The core histones were then adjusted to 0.1 M NaCl (final volume 100 μl) with 10 mM

Tris, 1 mM EDTA, 0.1% NP-40, 5 nM DTT, 0.5 mM PMSF, 20% (v/v) glycerol, and 100 μg/ml BSA. The mononucleosomes were purified on a 5–25% sucrose density gradient (10 mM HEPES, pH 7.5, 1 mM EGTA, 0.1 mM PMSF and 5% NP-40). Nucleosomes (200 μl) were layered on a 3.8 ml gradient and centrifuged at $33{,}000 \times g$ for 16 h. After fractionation the nucleosomes were stored at 4 °C.

2.6. DNase I footprinting

DNase I footprinting analysis was performed according to the procedure of Workman (Cote et al., 1995). After DNase I treatment of the mononucleosomes, reaction products were run on an 8% acrylamide-8M urea sequencing gel. The Thermo Sequenase Radiolabled terminator cycle sequencing kit for labeling single-stranded template DNA with [^{33}P] was obtained from USB (Cleveland, OH) and used according to the manufacturer's instructions.

2.7. EMSA analysis

Binding of ER to ERE or mononucleosomes was measured using a gel mobility shift assay. DNA or mononucleosomes were incubated without or with various concentrations of ER-containing nuclear extract or recombinant ERα for 20 min at room temperature. This mixture was then incubated with 500 ng poly d(I-C) for 15 min to bind nonspecific DNA binding proteins. After the addition of radiolabeled probe DNA or mononucleosomes, the mixture was incubated for another 15 min at room temperature. Reaction mixtures were loaded onto a 5% polyacrylamide (acrylamide:bisacrylamide, 29:1) gel-0.5 × TBE (450 mM Tris, 450 mM boric acid 1 mM EDTA, pH 8.3) and run at 300 V for 1.5 h in 0.5 × TBE. The gels were dried, and bands visualized by autoradiography.

3. Results

3.1. Purification of reconstituted mononucleosomes

The core histones H2A, H2B, H3 and H4 were isolated from MCF-7 cells and their concentration and purity confirmed by SDS-PAGE and acid-urea-triton (AUT) gel electrophoresis (not shown). After reconstitution with radiolabeled probe DNA the mononucleosomes were purified by sucrose density gradients and fractionated. This allowed separation of free radioactivity and free probe DNA from the mononucleosomes (Fig. 1A). The various fractions were then subjected to native polyacrylamide gel electrophoresis to confirm that mononucleosomes were indeed formed (Fig. 1B). Some nucleosome cores dissociate into naked DNA upon dilution in the gradient, which is apparent in the mononucleosome fractions 20–27. This has also been reported by others (Cote et al., 1995). To also confirm that mononucleosomes were formed, a sample of the pooled mononucleosome fractions was subjected to DNase I footprint analysis (Fig. 1C). With DNase I treatment the assembled probe showed the 10 bp repeat pattern of protection resulting from alternating accessibility of the minor groove to DNase I (Wechsler et al., 1994).

3.2. Binding of ER to probe DNA

Before determining the binding parameters of ER binding to mononucleosomes, we first used EMSA analysis to determine the binding of ER to the 164 bp probe DNA to confirm that ER, indeed, does bind with high affinity to this construct containing a centrally located ERE. Nuclear extracts from estradiol-treated MCF-7 cells were incubated with the ERE. A retarded band was detected (lane 3, Fig. 2A and C) which was eliminated when the radiolabeled ERE was incubated with excess unlabeled ERE (Fig. 2C, lane 4). Also, incubation of the ERE with control nuclear extract (low ER) did not result in a retarded band (Fig. 2A, lane 2). Incubation of the ERE with recombinant ERα resulted in a large retarded band doublet. The formation of the doublet is probably due to partially degraded recombinant ER in the commercial preparation. Incubation of the ERE with HMGB-2 alone (Fig. 2C, lane 2) resulted in no significant retarded band, suggesting that HMGB-2 did not bind the DNA. However, addition of HMGB-2 to the ER containing nuclear extracts resulted in a much larger retarded band, suggesting that HMGB-2 interacted with the ER and stabilized the ER:ERE complex prior to or during the EMSA. This retarded complex was eliminated with excess unlabeled ERE (Fig. 2C, lane 6).

3.3. Binding of ER to mononucleosomes

Nuclear extracts were obtained from MCF-7 cells treated with estradiol to obtain an ER preparation or in the absence of phenol red and in the presence of charcoal-stripped serum to obtain a receptor depleted nuclear extract. Nucleosomes were incubated with increasing concentrations of ER-containing nuclear extracts and EMSA analysis performed. A dose dependence in the interaction of nucleosomes with ER was observed with the formation of a retarded ER:nucleosome band (Fig. 3A). Control nuclear extracts (low ER) did not form an ER:nucleosome band. The ER also interacted with the free DNA present in the nucleosome preparation. The position of the ER:DNA complex was determined in separate experiments (not shown) and was found to migrate slightly slower than nucleosomes. To demonstrate that the nucleosome-shifted band contained ER in the complex, nuclear extract from estradiol-treated cells was incubated with an anti-ERα polyclonal antibody. Nucleosomes were than added and the EMSA analysis performed (Fig. 3B). Both the ER:DNA complex and the ER-nucleosome complex were inhibited from forming in the presence of the anti-ER antibody. We also demonstrated a dose dependence in the interaction of mononucleosomes with recombinant ER (Fig. 3C). Surprisingly, the interaction

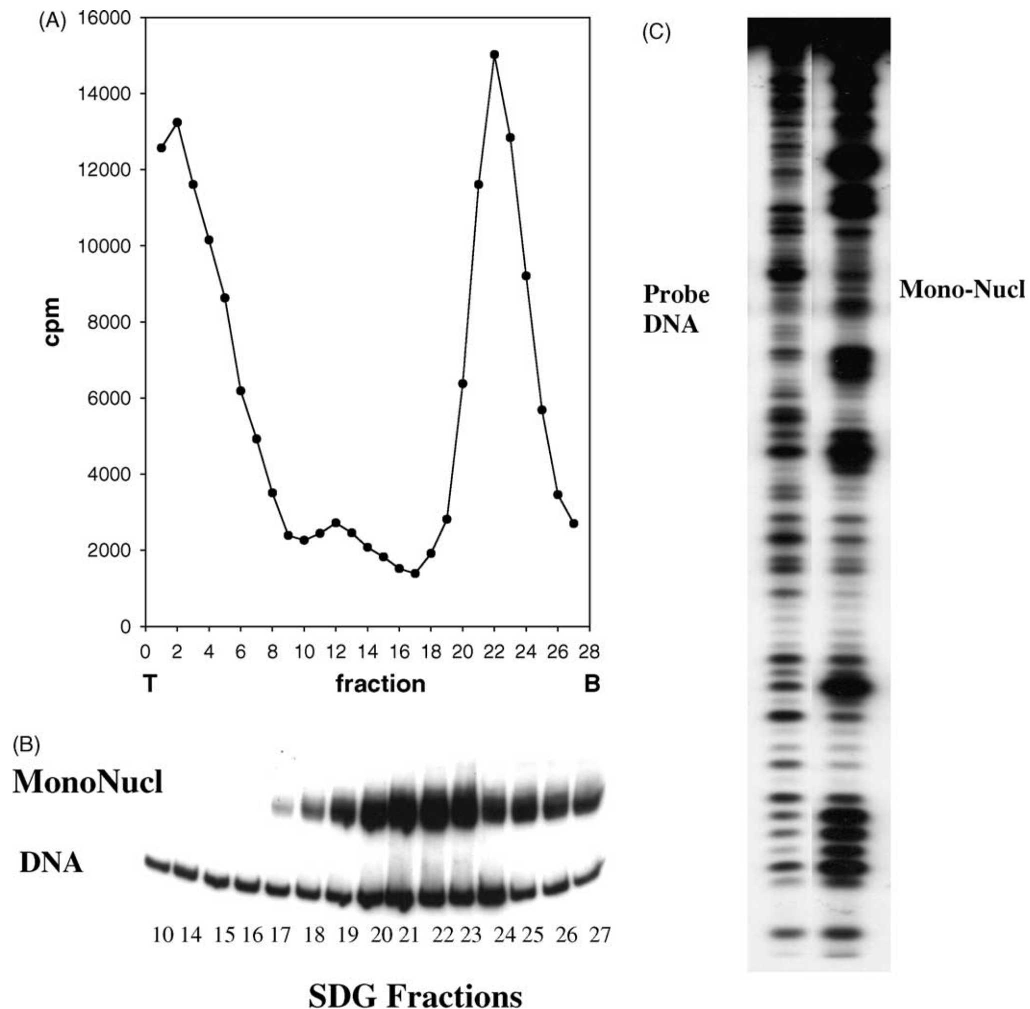

Fig. 1. Purification of reconstituted nucleosome core particles by sucrose density gradient centrifugation. Mononucleosomes were reconstituted as described in the Section 2. (A) [^{32}P]ERE-reconstituted mononucleosomes were purified by 5–25% sucrose density gradients, fractionated and counted. (B) Native polyacrylamide gel electrophoresis was used to determine the migration of free DNA from nucleosomes. (C) DNase I footprint analysis was performed with labeled free and nucleosomal DNA ± rERα or nuclear extract, respectively, to demonstrated the 10 bp repeat pattern of protection with mononucleosomes.

of the recombinant ERα with ERE in nucleosomal structure was much less than that for linear ERE (compare Fig. 2B with 3C). We also tested the interaction of the ER when bound by the triphenylethylene antiestrogen, H1285 (Ruh and Ruh, 1983), a compound structurally and functionally very similar to 4-hydroxy-tamoxifen. The results obtained were indistinguishable from ER bound by estradiol (not shown). Antiestrogen bound ER has also been shown to bind EREs similarly to estrogen bound ER (Kumar and Chambon, 1988).

3.4. HMGB-2 increases the interaction of ER with mononucleosomes

Since HMGB-1 or -2 have been shown to interact with both the estrogen and progesterone receptors (Boonyaratanakornkit et al., 1998; Melvin et al., 2002), and HMGB-2 interacts with the ER:ERE complex in Fig. 2C, we wanted to determine if HMGB-2 interacts with ER bound to ERE in nucleosomal structure. Nucleosomes were incubated with ER-containing nuclear extract (sub-optimal concentration) in the absence or presence of baculovirus expressed HMGB-2 (Fig. 4A). HMGB-2 alone did not bind ERE (lane 4); however, in the presence of ER containing nuclear extract, HMGB-2 appeared to stabilize the ER:nucleosomal structure (lanes 3 and 6). ER (10 μg nuclear extract) formed a tighter retarded band (Fig. 4A, lane 6) with HMGB-2 compared to half the concentration of ER that demonstrated a tendency for nucleosome dissociation during the EMSA (lane 3). In the absence of HMGB-2 the higher concentration of nuclear extract (lane 4) showed a greater tendency for interaction with mononucleosomes (indicated by the smear) than the lower dose (lane 2). However, in the absence of HMGB-2 this higher dose of nuclear extract could not maintain a stable receptor:nucleosome complex. The stabilization of ER binding in the presence of HMGB-2

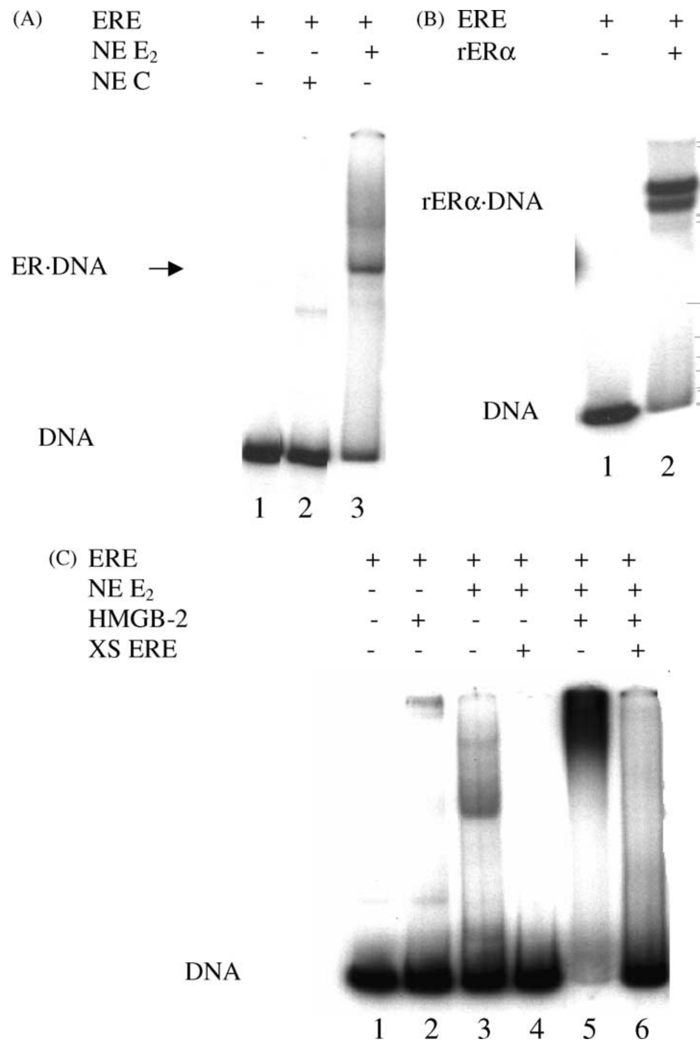

Fig. 2. ER binds specifically to a 164 bp ERE. EMSA analysis indicates that [^{32}P]ERE (164 bp) interacts with (A) nuclear extracts (7.5 μg) from MCF-7 cells treated with estradiol (NE E_2), but not with untreated cells (NE C) and with (B) recombinant ERα (rERα) (1.2 μg). (C) Incubation with 10-fold excess ERE (XS ERE, lane 4) inhibits binding of ER with the radiolabeled ERE (lane 3). ERE was also incubated with 5 μg nuclear extract from estradiol-treated MCF-7 cells in the absence of HMGB-2 (lane 3) or in the presence of 5 μg baculovirus expressed and purified HMGB-2 (lane 5). HMGB-2 was also incubated with ERE in the absence of nuclear extract (lane 2). Whereas HMGB-2 alone did not interact with ERE, HMGB-2 increased the formation of the ER-DNA complex. All data are representative of at least three individual experiments.

demonstrated a dose dependence of HMGB-2 (Fig. 4B) and addition of unrelated proteins such as bovine serum albumin had no effect (not shown). Incubation of the nucleosomes with excess ERE inhibited the interaction of ER with nucleosomes in the presence or absence of HMGB-2 (Fig. 4C, lanes 2 and 4). Incubation of anti-HMGB-2 with the ER and nucleosomes did not shift the ER:nucleosomal band (not shown). Since HMGB-2 did not bind directly to the DNA but appears to bind ER and stabilize the ER:nucleosomal structure, the inability to observe a further shift with anti-HMGB-2 might suggest that HMGB-2 dissociated from the complex prior to or during electrophoresis. Nucleosomes were also incubated with recombinant ERα in the presence or absence of HMGB-2 (Fig. 4D). As seen in Fig. 3, recombinant ERα only shifted a portion of the nucleosomes. However, the ER:nucleosome complex was stabilized in the presence of HMGB-2, forming preferentially the higher molecular weight ER:nucleosome complex. It is interesting that ER in nuclear extracts in combination with HMGB-2 shifts the entire nucleosome band, whereas recombinant ERα which is present in a much greater concentration compared to ER in nuclear extracts, shifts only a portion of the nucleosome band. This suggests that there is another protein(s) in the nuclear extract that aids in the stabilization of the ER:nucleosome complex.

In order to further determine how ER affects nucleosomal structure we performed DNase I footprinting. Whereas recombinant ERα protected the ERE from digestion (Fig. 5A), nuclear extracts containing ER did not appear to protect nucleosomes in the ERE region (Fig. 5B). The nucleosome digestion pattern, which differs significantly from DNA (compare Fig. 5A and B), was altered in the presence of

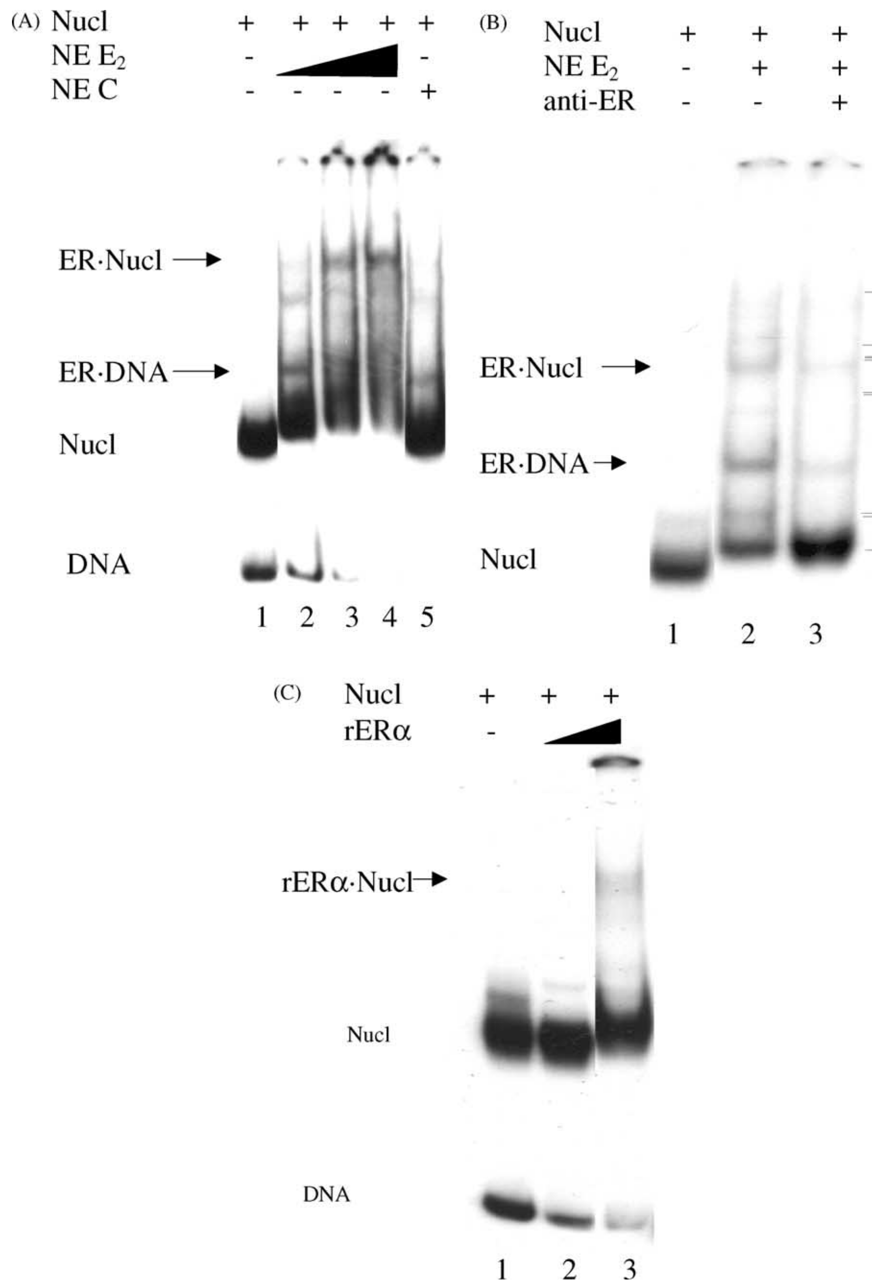

Fig. 3. ER binds to mononucleosomes. EMSA analysis (5% acrylamide gel) was developed to investigate the interaction of ER with purified mononucleosomes (approximately 2000 cpm per reaction). (A) A dose dependence in the interaction of nucleosomes (Nucl) with increasing concentrations (lanes 2–4: 7.5, 12, and 15 μg, respectively) of nuclear extract from estradiol-treated MCF-7 cells (NE E_2) was observed. Nuclear extracts (12 μg) from MCF-7 cells grown in phenol red free medium (NE C) did not demonstrate a significant ER interaction with nucleosomes (lane 5). (B) ER-containing nuclear extract was incubated with a anti-ERα polyclonal antibody for 2 h at 4 °C. Nucleosomes were then added and the EMSA analysis performed. Both the ER:DNA complex and the ER:nucleosome complex were inhibited from forming in the presence of the anti-ER (lane 3). (C) A dose dependence with recombinant ERα (rERα) was also observed (lane 2: 0.3 μg; lane 3: 9 μg). All data are representative of at least 4–5 individual experiments except for (B) which was performed twice.

ER (see arrows). While there was no protection seen in the region where nucleosomes contained the ERE, cleavage was actually enhanced in certain regions. Sites of enhanced cleavage in nucleosomal DNA have been reported for Max binding to nucleosomes (Weschler et al., 1994). There were also sites of decreased cleavage. Addition of HMGB-2 did not change the DNase I digestion pattern from that seen in the presence of ER alone, suggesting that HMGB-2 stabilizes the ER:nucleosomal complex without altering the formation of the nucleosomal structure. DNase I digestion of nucleosomes in the presence of recombinant ERα gave results that were not readily interpretable since recombinant ER only interacts with a small portion of the nucleosomes under the conditions of our assay, thus the digestion pattern

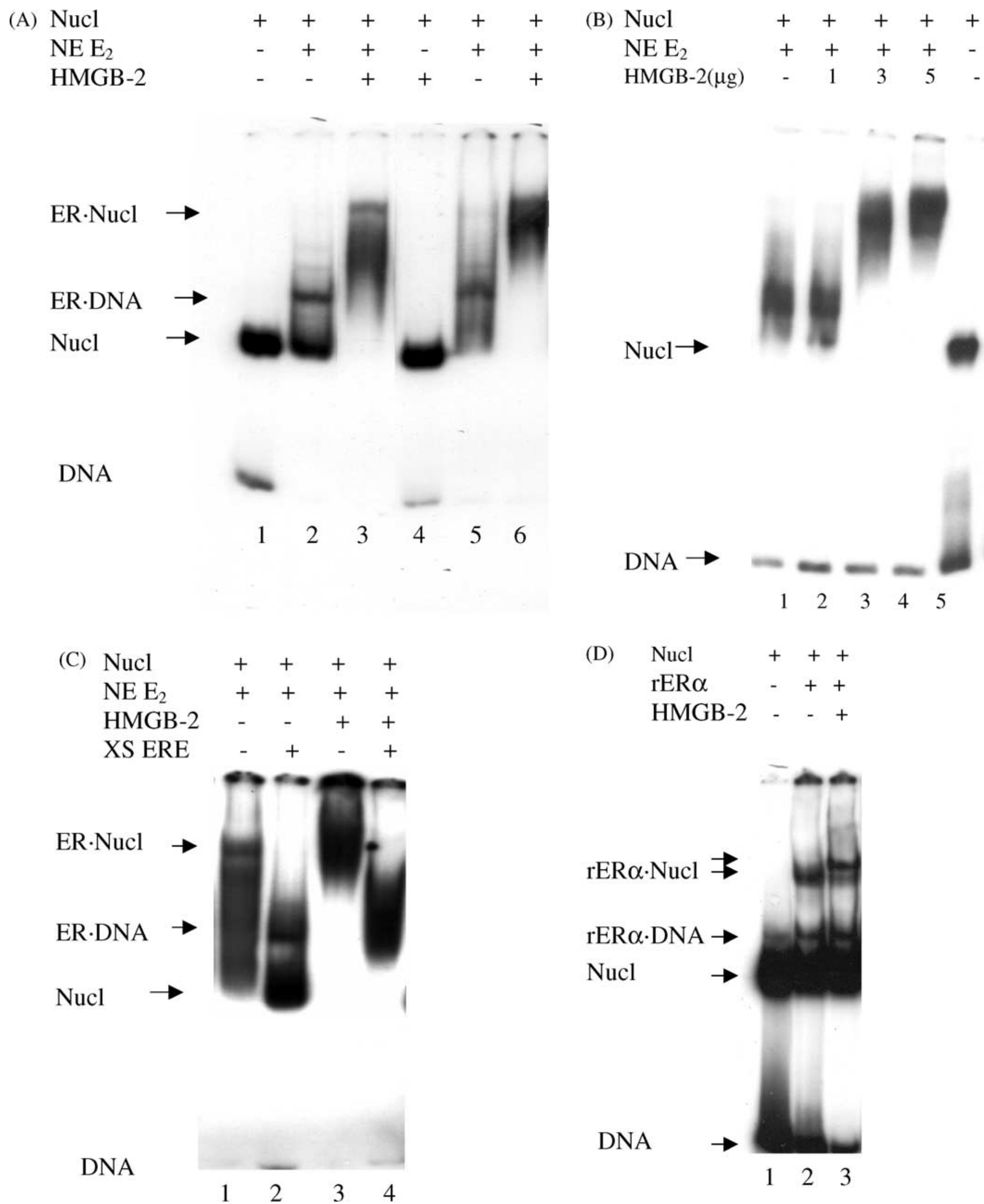

Fig. 4. HMGB-2 increases the interaction of ER with nucleosomes. (A) Nucleosomes were incubated with 5 μg (lanes 2 and 3) or 10 μg (lanes 5 and 6) nuclear extract from estradiol-treated MCF-7 cells in the absence of HMGB-2 or in the presence of 5 μg baculovirus expressed and purified HMGB-2 (lanes 3 and 6). HMGB-2 was also incubated with nucleosomes in the absence of nuclear extract (lane 4). Whereas HMGB-2 alone did not interact with nucleosomes, HMGB-2 increased the formation of the ER-nucleosome complex. (B) Varying amounts of HMGB-2 were added to the nucleosomes plus nuclear extract. (C) Excess ERE (100-fold, XS ERE) inhibited the interaction of ER-containing nuclear extract with nucleosomes in the presence or absence of HMGB-2. (D) Nucleosomes were also incubated with recombinant ER (5 μg) in the absence or presence of 5 μg HMGB-2. Addition of HMGB-2 caused a shift in the nucleosomal band. The data are representative of three individual experiments.

mostly reflected DNase digestion of nucleosomes in the absence of ER (not shown).

4. Discussion

There are numerous studies describing the interaction of the ER with consensus sequence EREs; however, this is the first report of the interaction of ER with an ERE in nucleosomal structure. We prepared mononucleosomes from core histones and a linearized DNA with a vitellogenin ERE that is centered in the DNA. The ERE in this structure more closely resembles the DNA constrained within the chromatin architecture. The major focus of the present study was to determine the binding characteristics of ER to the ERE in nucleosomal structure. We observed that the binding of ER to mononucleosomes demonstrated a dependence on amount of ER and that the binding was specific. Addition

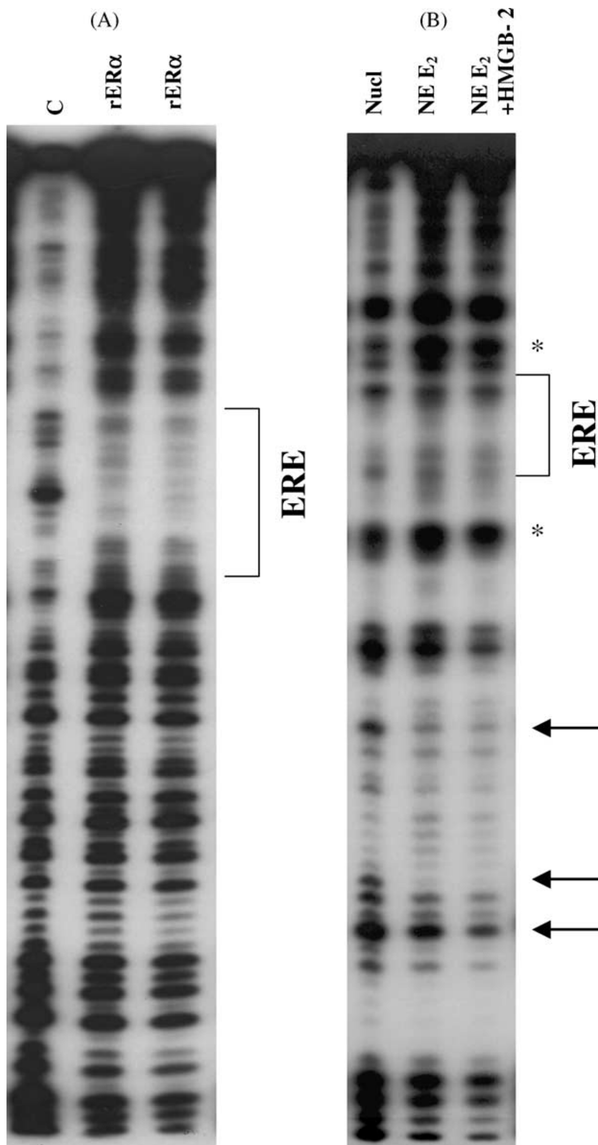

Fig. 5. DNase I footprint analysis of ER binding to probe DNA is different from binding to mononucleosomes. (A) DNase I footprint with labeled probe DNA (ERE) in the absence or presence of 2 and 4 μg recombinant human ERα. After DNase treatment reaction products were run on an 8% acrylamide-8M urea sequencing gel. The bracket indicates the position of the consensus ERE and protected sites. (B) DNase I footprint with labeled mononucleosomes in the absence or presence of 20 μg ER-containing nuclear extract plus or minus 10 μg HMGB-2. Arrows indicate sites of enhanced cleavage of DNA bases with addition of ER ± HMGB-2 vs. absence of ER. The symbol (*) indicates sites of decreased cleavage.

of an anti-ER antibody to the ER preparation prior to addition of the mononucleosomes inhibited the binding of ER to the ERE and nucleosomes. These results were in many ways similar to our studies of the binding of ER from either estradiol-treated MCF-7 cell nuclear extracts or recombinant ERα to the linearized 164 bp ERE which displayed the characteristics typical of the binding of ER to an ERE. The binding characteristics were dependent on the amount of ER present in the reaction and an excess amount of unlabeled ERE competed with the radiolabeled ERE. Nuclear extract from non-stimulated MCF-7 cells showed minimal binding to the ERE.

Several highly purified receptors of the steroid receptor superfamily demonstrate weak binding affinity for their respective hormone response elements. Although the ER DNA binding domain interacts weakly with various palindromic EREs in the absence of added nuclear factors, it has been reported that addition of HMGB-1significantly increased the binding affinity of ER for ERE (Romine et al., 1998; Zang et al., 1999). This enhancement appeared to be due in part to a decreased dissociation of the ER DBD from the ERE. Highly purified recombinant progesterone receptor (PR), on the other hand, failed to bind a GRE/PRE in the absence of HMGB-1 or -2 (Melvin et al., 2002). Verrijdt et al. (2002) reported that various steroid receptor DBDs have increased affinity for the GRE in the presence of HMGB-1, but HMGB-1 had no effect on DNA binding by the various receptors. Thus, although HMGB-1/2 is reported to bind with low affinity and in a sequence independent manner to double stranded DNA, there is no evidence that HMGB-1/2 specifically binds hormone response elements directly. Our data demonstrated no binding of HMGB-2 to the probe DNA containing the ERE or to this DNA in nucleosomal structure. This suggests that HMGB-2 associates with the ER to stabilize the ER:ERE interaction. This is supported by studies with a monoclonal anti-HMGB-1 antibody, 854E10, where only a very small portion of the receptor:ERE complex could be supershifted in a gel mobility shift assay (Romine et al., 1998), suggesting that the HMGB protein dissociates prior to or during the electrophoretic run.

A transcriptional activator, such as ER, that seeks to bind nucleosomal DNA must contend with the relatively constrained structure of the DNA while wrapped around the core histones to form the nucleosome. Therefore, our present studies were undertaken to characterize the ER:ERE interaction while the DNA is in nucleosomal structure. Interestingly, the interaction of ER for the nucleosome was less than for linearized DNA. Within the nucleus, ER must initially interact with nucleosomal DNA prior to disruption or unwinding of the nucleosome. However, as seen with ERE in a longer DNA probe, we also report that HMGB-2 stabilizes the ER bound to mononucleosomes. A monoclonal antibody to HMGB-2 failed to cause a supershift, again suggesting a dissociation of HMGB-2 prior to or during the gel electrophoresis. The ER nucleosome complex formed was specific since excess unlabeled ERE inhibited the formation of a radiolabeled retarded band. While one group (Verrier et al., 1997) reported that order of addition of HMGB-1 to ER and ERE affected the ability of HMGB-1 to enhance ER binding to DNA, others (Romine et al., 1998) have found that order of addition of HMGB-1 had no significant effect. Our studies with HMGB-2 with ERE or ERE in nucleosomal structure demonstrated no significant difference with order of addition of components of the assay.

In addition to enhancing binding of specific DNA binding proteins to DNA, HMGB-1/2 has also been reported to enhance transcriptional activity of several transcription factors, including steroid hormone receptors (Melvin and Edwards, 1999; Verrijdt et al., 2002). Although HMGB-1/2 have been reported to enhance binding of several classes of transcription factors, there is some specificity since HMGB-1/2 do not enhance DNA binding of nuclear receptors that heterodimerize with RXR (Boonyaratanakornkit et al., 1998). It is not known whether HMGB-1 and -2 have distinct functions or are redundant gene products. In previous studies regarding steroid hormone receptor binding to DNA HMGB-1 and -2 were interchangeable (Melvin and Edwards, 1999), therefore in our studies we only investigated the effect of HMGB-2 on ER interaction with nucleosomes. The mechanism by which HMGB-1/2 enhances the binding of select steroid hormones to their respective response elements appears to involve the C-terminal extension (amino acid sequences adjacent to the zinc finger core DBD) of these receptors (Melvin et al., 2002). These HMGs transiently bind the receptor C-terminal extension altering the conformation of the receptor resulting in an increase in DNA binding affinity. This hypothesis is supported by findings that HMGB-1/2 does not bind EREs or PREs directly, or, as reported here, ERE in nucleosomes. Boonyaratanakornit et al. (1998) reported a weak interaction between PR and HMGs, but not with nuclear receptors that partner with RXR. Therefore, steroid hormone receptors such as ER must be in proper conformation resulting from both ligand binding and HMGB interaction in order to become part of a nucleosome complex, the unit that coactivators and transcription factors must initially interact with to affect modifications in chromatin structure associated with gene activation.

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